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Discovery of FabH/FabF Inhibitors from Natural Products

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Condensing enzymes are essential in type II fatty acid synthesis and are promising targets for antibacterial drug discovery. Recently, a new approach using a xylose-inducible plasmid to express antisense RNA in *Staphylococcus aureus* has been described; however, the actual mechanism was not delineated. In this paper, the mechanism of decreased target protein production by expression of antisense RNA was investigated using Northern blotting. This revealed that the antisense RNA acts posttranscriptionally by targeting mRNA, leading to 5′ mRNA degradation. Using this technology, a two-plate assay was developed in order to identify FabF/FabH target-specific cell-permeable inhibitors by screening of natural product extracts. Over 250,000 natural product fermentation broths were screened and then confirmed in biochemical assays, yielding a hit rate of 0.1%. All known natural product FabH and FabF inhibitors, including cerulenin, thiolactomycin, thiotetromycin, and Tü3010, were discovered using this whole-cell mechanism-based screening approach. Phomallenic acids, which are new inhibitors of FabF, were also discovered. These new inhibitors exhibited target selectivity in the gel elongation assay and in the whole-cell-based two-plate assay. Phomallenic acid C showed good antibacterial activity, about 20-fold better than that of thiolactomycin and cerulenin, against *S. aureus*. It exhibited a spectrum of antibacterial activity against clinically important pathogens including methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, and *Haemophilus influenzae*.

Hundreds of essential proteins have been identified in bacteria as potential drug targets (1, 16, 18, 23). Of these, only a few are targets of therapeutically useful drugs. These include penicillin binding proteins, D-Ala-D-Ala ligase, MurA, undecaprenyl pyrophosphate, and alanine racemase for cell wall; 30S and 50S ribosomal subunits, elongation factor G, and Ile-tRNA synthetase for protein synthesis; RNA polymerase for RNA synthesis; InhA (FabI) for fatty acid synthesis; dihydrofolate reductase (FolA) and *p*-aminobenzoic acid synthase (FolP) for metabolism; and DNA gyrase and topoisomerase IV for DNA synthesis. In past decades, extensive chemical modification of existing antibiotics has afforded improved activity against their targets. This strategy served well to develop new and effective antibiotics; however, such modification is becoming increasingly difficult and identification of new classes of compounds with different modes of action is critical to combat emerging resistance and meet clinical needs.

Type II fatty acid synthesis (FASII) is essential to bacterial cell viability. The significant differences in organization, structure of enzymes, and role played by fatty acids between bacteria and humans make this system an attractive target for antibacterial drug discovery (3, 11, 12, 42, 48, 51). One essential enzyme, FabI, in the fatty acid synthesis pathway is a proven drug target. Two marketed antibacterial agents that target the FabI enzyme are triclosan (antiseptic) and isoniazid (an anti-*Mycobacterium tuberculosis* agent) (2, 13). An initiation condensing enzyme, FabH, and elongation condensing enzymes, FabF/B, are also essential enzymes in this pathway (26, 37, 39, 43), and they are highly conserved among key pathogens. Although no drugs targeting condensing enzymes are used in the clinic, two natural products, cerulenin (28) and thiolactomycin (30), which selectively inhibit the condensation enzymes FabH and FabF/B, were discovered more than 2 decades ago. Cerulenin selectively targets FabF/B and forms a covalent bond with the cysteine in the active site of FabF/B with its tail occupying the long hydrophobic cavity that normally contains the growing acyl chain of the natural substrate (21, 34). Thiolactomycin and its analogs (6, 31) inhibit both FabH and FabF/B and bind to the malonate portion of the active site. Besides cerulenin and thiolactomycin, several fatty acid synthesis inhibitors targeting condensing enzymes have been identified using in vitro biochemical approaches; however, most of them either failed to reach intracellular targets due to poor penetration or lacked target selectivity in whole cells. In a previous study, an approach for evaluating fatty acid synthesis inhibitors in vitro and in vivo was described, which includes a FASII pathway assay, a whole-cell labeling assay, and a test of cell penetration and efflux in addition to determination of the spectrum of antibacterial activity (24). In the present study, the mechanism of action of antisense RNA (AS-RNA) in regulating FabH/FabF enzyme expression is addressed and the development of a cell-based FabH/FabF-specific, agar-diffusion two-plate differential sensitivity assay is described. Over 250,000 natural product extracts from actino-

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mycetes and fungi were screened and confirmed through secondary biochemical assays leading to identification of several FabH/FabF inhibitors. These inhibitors showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis*, and *Haemophilus influenzae* with target selectivity for FabF/H in both biochemical and whole-cell assays.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma-Aldrich Chemical Co. unless otherwise indicated. Miller's LB broth (Invitrogen; 12795-027), select agar (Invitrogen; 30391-023), bioassay dish (Nunc; 240385), dialysis tubing (Invitrogen; 15961-022), *Haemophilus* test medium (Remel; REF 112380), dithiothreitol (DTT; Fisher; BP172-5), β-mercaptoethanol (Bio-Rad; 161-0710), and RNA ladders (Invitrogen; 15620-016 and 15623-010) were used in this study. Radiolabeled chemicals were from Perkin-Elmer (NEN) Life Sciences. Acyl carrier protein (ACP; Sigma-Aldrich Chemical Co.; A7303) was pretreated with 3 mM DTT on ice for 20 min, aliquoted, and stored at −80°C.

Agar-diffusion two-plate differential sensitivity assay (two-plate assay). *S. aureus* cells (RN450) carrying plasmid S1-1941 bearing antisense (1941A) to *fabF* (*fabF* AS-RNA strain) or vector (control strain) (8) were inoculated in Miller's LB broth containing 34 μg/ml of chloramphenicol and incubated overnight at 37°C with shaking at 220 rpm. Each culture was diluted to a final optical density at 600 nm of 0.003 into a flask containing Miller's LB broth supplemented with 1.2% select agar (autoclaved and cooled to 48°C), 0.2% glucose, 15 μg/ml chloramphenicol, and 50 mM of xylose. Two assay plates, one seeded with the *fabF* AS-RNA strain (AS plate) and the other seeded with the control strain (control plate), were prepared by pouring 100 ml of each of the above mixtures into a 20-cm-by-20-cm bioassay dish. Immediately, well casters were placed into the agar and the agar was allowed to solidify at room temperature for 30 min. The samples (20 μl) were applied to wells on both the control and AS plates and were incubated at 37°C for 18 h. Any difference in zone size between the AS plate and the control plate was measured in millimeters, with an active sample showing a larger zone on the AS plate.

FASII and gel elongation assays. The details of the preparation of FASII enzymes from *Escherichia coli*, *S. aureus*, or *B. subtilis* were described previously (24). Preparation of FASII enzymes from *H. influenzae* is similar to that of those from *E. coli*. Briefly, cells were grown to stationary phase in *Haemophilus* test medium and centrifuged at 8,000 rpm for 10 min using a Beckman JA-10 rotor. The pellets were washed twice with ice-cold buffer A (0.1 M sodium phosphate, pH 7, 1 mM EDTA, and 5 mM β-mercaptoethanol) and resuspended in the same buffer plus 0.1 mg/ml lysozyme. The cells were lysed on ice for 30 min, sonicated five times in 1-min bursts in an ice water bath, and centrifuged at 20,000 rpm for 15 min at 4°C using a Beckman JA-20 rotor. The supernatant was collected and precipitated with ammonium sulfate. The pellet from the 40 to 85% ammonium sulfate fraction was resuspended in buffer A and dialyzed at 4°C against four changes of the same buffer using 10-kDa-molecular-mass-cutoff dialysis tubing. The protein was aliquoted, flash frozen using liquid nitrogen, and stored at −80°C. Protein concentration was determined using the standard Bio-Rad protocol. The FASII assay was performed in a phospholipid 96-well Flashplate as described previously (24). Briefly, 3 μg FASII enzymes (except for *S. aureus*, which used 1.26 μg) was preincubated with inhibitors at room temperature for 20 min in 50 μl buffer containing 100 mM sodium phosphate (pH 7.0), 5 mM EDTA, 1 mM NADPH, 1 mM NADH, 150 μM DTT, 5 mM β-mercaptoethanol, 20 μM *n*-octanoyl coenzyme A (CoA) (or lauroyl-CoA), 4% Me_2SO, and 5 μM of the pretreated ACP. The reaction was initiated by addition of 10 μl of water-diluted [2-^{14}C]malonyl-CoA (60 mCi/mmol), which gave a final concentration of 4 μM malonyl-CoA with total counts of about 20,000. The reaction mixture was incubated at 37°C for 30 min for *E. coli* and 90 min for *S. aureus*, *B. subtilis*, and *H. influenzae*. The reaction was terminated by adding 100 μl of 14% perchloric acid. The plates were sealed and counted for 5 min using a Packard TopCount NXT scintillation counter. The gel elongation assay was done in conditions identical to those for FASII with some exceptions. The reactions were carried out in 1.5-ml Eppendorf tubes (polypropylene) and were terminated by flash freezing or direct application of the reaction mixtures to urea-polyacrylamide gels as described previously (24) and in the figure legends. All data were analyzed using Prism (GraphPad Software, Inc.).

RNA purification and Northern blot analysis. *S. aureus* RN4220 strains containing *fabF* antisense RNA (696A, 1941A, 1949A, and 1961A) were grown to mid-log phase in LB broth containing 34 μg/ml chloramphenicol at 37°C. Xylose induction was performed by addition of 20 mM xylose for 5 min, and the cells were centrifuged at 4,100 rpm for 2 minutes just before RNA isolation. RNA was purified from each culture using a modified version of the RNeasy 96 RNA purification system (QIAGEN), and the concentration and purity were determined by readings of optical density at 260/280 nm. Total RNA (1 μg) was denatured at 55°C for 10 min in RNA sample loading buffer and was analyzed on a 1% agarose gel containing 2% formaldehyde as described previously (46). Probe 1 (5′-CAATATCCAAATCACATTCTGGGTCTGGTGTTACCGCAT-3′), probe 2 (5′-TTGTGCTGATTCTAAAGATTCGATTACTAAAATACCAGCA-3′), and probe 3 (5′-AATCCAGAACATGCTGCAAGTTGATCCATAGAGGCA ACTT-3′) were labeled with [^{32}P]dATP using the StarFire kit (Integrated DNA Technologies). The blots were probed at 68°C in Rapid-Hyb buffer (GE Healthcare), washed twice at 65°C for 20 min in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate, and visualized with a PhosphorImager.

Isolation of compounds. The seed culture of *Phoma* sp. was prepared by inoculation from frozen mycelium agar plugs in a 250-ml Erlenmeyer flask containing 60 ml of KFA seed medium of the following composition (grams/liter): corn steep powder, 2.5; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; agar, 4.0; and trace elements solution, 10 ml [in grams/liter, $FeSO_4 \cdot H_2O$, 1.0; $MnSO_4 \cdot 4H_2O$, 1.0; $CuCl_2 \cdot 2H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.019; $ZnSO_4 \cdot 7H_2O$, 0.2]. The pH of the medium was adjusted to 6.8 by addition of NaOH. The seed culture was incubated at 22°C on a gyratory shaker (220 rpm) for 5 to 8 days. The pH of the seed culture was 5.8. The production medium (CYS80) was prepared in 500-ml unbaffled flasks with 120 ml of medium. CYS80 was formulated as follows (grams/liter): sucrose, 80; cornmeal (yellow), 50; and yeast extract, 1. No pH adjustment was made prior to inoculation. Fermentation flasks were inoculated with 4% vegetative seed growth and were incubated at 22°C at 220 rpm and 70% humidity for 14 days. The final pH of the fermentation broth was 4.4. One liter of the fermentation broth was extracted with 1 liter of ethyl acetate and then concentrated to dryness under reduced pressure to produce 1 g of oil. This material was dissolved in 10 ml methanol, applied to to a 550-ml Sephadex LH20 column (2.54 × 500 cm), and eluted with methanol at 5 ml/min. Ten milliliters of each fraction was collected. The active compounds eluted from fractions 31 to 35. These fractions were pooled and concentrated to dryness, yielding 132 mg of a gum which was dissolved in 1.2 ml methanol and analyzed on a reverse-phase high-pressure liquid chromatograph (Atlantis; 30 × 100 mm). Elution of the column at a flow rate of 10 ml/min with a 50-min linear gradient of 25 to 80% aqueous acetonitrile afforded phomallenic acid A at 21 min, B at 34 min, and C at 38 min.

MIC. The MIC against each of the strains was determined as previously described (45).

RESULTS

Investigation of *fabF* antisense mechanism. Four *fabF* AS-RNAs were designed and inserted into a xylose-inducible vector (8). Genetic analysis of the *S. aureus* genome using operon and transcription terminator prediction methods (47) showed that *fabH* is upstream of *fabF* and possibly both are members of the same operon (Fig. 1A). This hypothesis was confirmed by Northern blotting using three different probes (probes 1 and 2 for *fabF* and probe 3 for *fabH*). As predicted, the full-length mRNA transcripts showed identical sizes (~2,265 bases) with all probes (Fig. 1B, C, and D, lanes without induction). From Fig. 1B, with xylose induction of 696A, 1941A, 1949A, and 1961A antisense RNAs, the full-length mRNAs were not observed, which might be due either to antisense RNAs interfering with transcription machinery or to mRNA degradation by RNases. To further understand the mechanism of the antisense RNA action, a Northern blot assay was performed using probe 1 on all four antisense strains, which resulted in one shorter transcript from all strains with barely detectable full-length mRNA (Fig. 1C, lanes with induction). Because probe 1 is located at the 3′ portion of mRNA, the short bands must be 3′ portions of the transcript around probe 1. The sizes of these short bands of mRNA from strains expressing 696A, 1941A, 1949A, or 1961A antisense RNA showed ~750, ~850, ~1,000, or ~280 bases, respectively. These sizes seemed to match with sizes from the start of the antisense to the 3′ end of the mRNA,

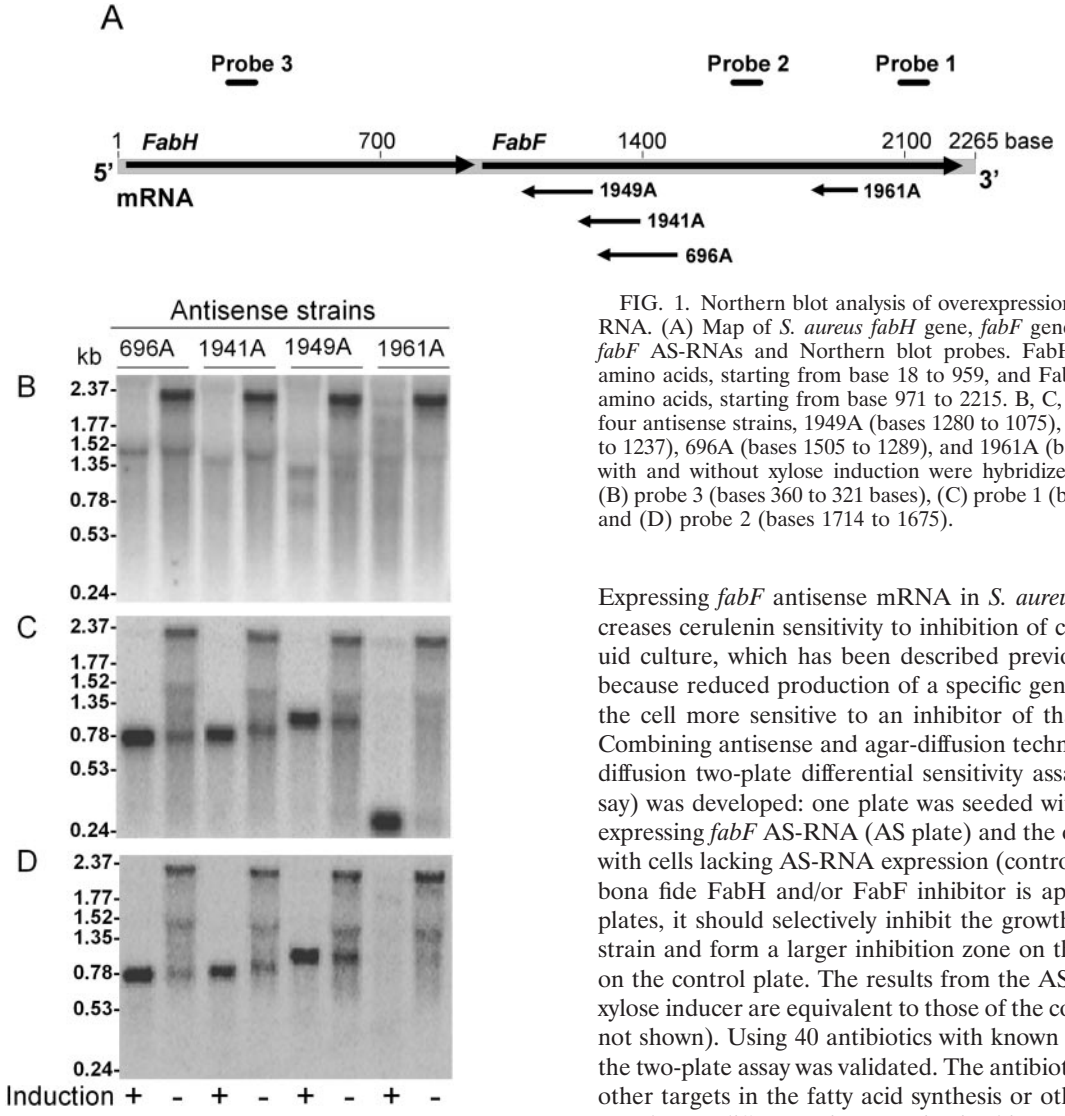

FIG. 1. Northern blot analysis of overexpression of *fabF* antisense RNA. (A) Map of *S. aureus fabH* gene, *fabF* gene, and locations of *fabF* AS-RNAs and Northern blot probes. FabH protein has 313 amino acids, starting from base 18 to 959, and FabF protein has 414 amino acids, starting from base 971 to 2215. B, C, and D. Blots from four antisense strains, 1949A (bases 1280 to 1075), 1941A (bases 1409 to 1237), 696A (bases 1505 to 1289), and 1961A (bases 1986 to 1860), with and without xylose induction were hybridized with ^{32}P-labeled (B) probe 3 (bases 360 to 321 bases), (C) probe 1 (bases 2111 to 2073), and (D) probe 2 (bases 1714 to 1675).

implying that the 3′ portion is stable and remains intact, while selective degradation occurs around the antisense targeting area. To confirm this observation, a Northern blot assay was performed using probe 2, which is located between the 1961A and 696A antisense targeting sites (Fig. 1D). The result showed the absence of the band of ~280 bases or of other longer bands for the strain expressing 1961A antisense with all other results remaining consistent with those using probe 1. This indicated that the ~280-base band was indeed the 3′ portion of mRNA and the 5′ portion was degraded.

Validation of two-plate assay. In general, when an antibiotic is applied to a well of an agar plate seeded with growing bacterial cells, it diffuses into the agar, forming a concentration gradient. Close to the well, the concentration is high enough to inhibit growth, while further from the well, a low-enough concentration is reached to allow growth of cells. Hence, a clear, circular zone is formed around the well due to the inhibition of cell growth. The zone size depends on the dose, potency, and diffusion rate of inhibitors and the growth rate of the bacteria. Expressing *fabF* antisense mRNA in *S. aureus* specifically increases cerulenin sensitivity to inhibition of cell growth in liquid culture, which has been described previously (8). This is because reduced production of a specific gene product makes the cell more sensitive to an inhibitor of that gene product. Combining antisense and agar-diffusion technologies, an agar-diffusion two-plate differential sensitivity assay (two-plate assay) was developed: one plate was seeded with *S. aureus* cells expressing *fabF* AS-RNA (AS plate) and the other was seeded with cells lacking AS-RNA expression (control plate). When a bona fide FabH and/or FabF inhibitor is applied to the two plates, it should selectively inhibit the growth of the *fabF* AS strain and form a larger inhibition zone on the AS plate than on the control plate. The results from the AS plate minus the xylose inducer are equivalent to those of the control plate (data not shown). Using 40 antibiotics with known modes of action, the two-plate assay was validated. The antibiotics that inhibited other targets in the fatty acid synthesis or other pathways did not show a difference in zone size in this assay.

Screening of natural products and isolation of FabH/F inhibitors. Over 250,000 natural product extracts were screened with the two-plate assay at a single concentration, giving a hit rate of 0.3% (0.1% when combined with biochemical assays). As an example (Fig. 2), 40 samples together with a serial dilution of cerulenin and thiolactomycin as controls were applied at identical positions on the control and *fabF* AS-RNA plates. Most natural product extracts showed antibacterial activity, with zones of inhibition without any zone differential between the two plates except for B6, which showed a zone diameter of 24 mm on the *fabF* AS-RNA plate compared to a zone diameter of 9 mm on the control plate. To confirm that the sample in B6 contained genuine fatty acid inhibitors, it was titrated in the FASII assay using *S. aureus* enzymes (Fig. 3). Cerulenin showed a 50% inhibitory concentration (IC_{50}) of 1.5 μg/ml, and the sample B6, an acetone extract of fungal broth, showed an IC_{50} of 12.4 μg/ml. From this FabH/F active broth, three phomallenic acids (designated A, B, and C) were isolated which account for both antibacterial and FASII activities. The structures of phomallenic acids are shown in Fig. 4. The details of isolation and structure elucidation will be published else-

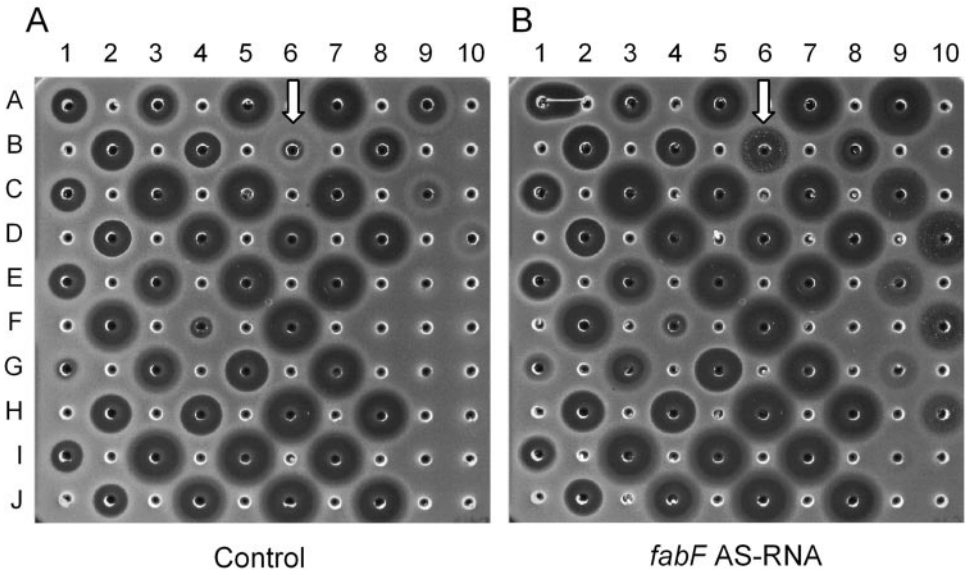

FIG. 2. Screen of natural product extracts. Two plates were prepared as described in Materials and Methods. Each natural product (20 μl) from actinomycetes and fungal fermentation broths, as well as controls, was applied into a well on both plates at identical positions. Thiolactomycin (1, 0.5, 0.25, and 0.125 mg/ml in rows 9A, 9C, 9E, and 9G, respectively) and cerulenin (1, 0.5, and 0.25 mg/ml in rows 10D, 10F, and 10H, respectively) were used as positive controls. The active broth 6B (indicated by arrows) showed a larger zone on the *fabF* AS-RNA plate, with a diameter of 24 mm as opposed to that on the control plate with a 9-mm diameter. This resulted in a zone differential of 15 mm, signaling a selective FabH/F inhibitor. Position 1A in panel B shows an irregular zone due to a crack in the agar resulting in uneven diffusion of the inhibitor (in this case, the sample was reconfirmed). All other samples display antibacterial activity but no significant zone differential.

where (31a). In this screen, cerulenin, thiolactomycin, thiotetromycin (31), and Tü3010 (36) were identified multiple times. Cerulenin is produced by fungal sources, and the last three compounds are produced by *Streptomyces*. The structures and their activities against *S. aureus* are shown in Fig. 5. Cerulenin is a covalent inhibitor of FabF/B, and the correlation of its FASII IC_{50} and antibacterial activity has been described previously (24). Among the three analogs of thiolactomycin, Tü3010 had the best antibacterial activity, >15-fold better than the other two compounds. The inhibition of FASII (IC_{50} values) was similar for all three compounds, suggesting that Tü3010 potentially has better cell penetration. Tü3010 is a natural carboxamide analog of thiotetromycin. While the antibacterial activity of this compound was previously published (36), the fatty acid synthesis inhibitory activity is reported here for the first time.

Evaluation of phomallenic acids. Phomallenic acids A, B, and C exhibited good antibacterial and FASII activities against *S. aureus*, *H. influenzae*, *B. subtilis*, and *E. coli* (Table 1). Phomallenic acids are acetylenic allene versions of fatty acids. Phomallenic acid A is the smallest of the three phomallenic

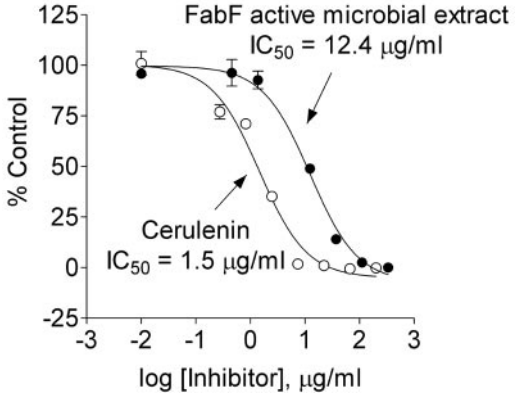

FIG. 3. FASII assay on the FabH/FabF active broth. The FASII assay was performed with a serial dilution of FabH/F active acetone extract (●) (333- to 0.01-μg/ml final concentration) and cerulenin (○) (200- to 0.01-μg/ml final concentration) using *S. aureus* FASII enzymes, providing IC_{50} values of 12.4 μg/ml (95% confidence interval, 8.7 to 17.6) and 1.5 μg/ml (95% confidence interval, 0.97 to 2.2), respectively. The graph shows the results of an average of two duplicate experiments.

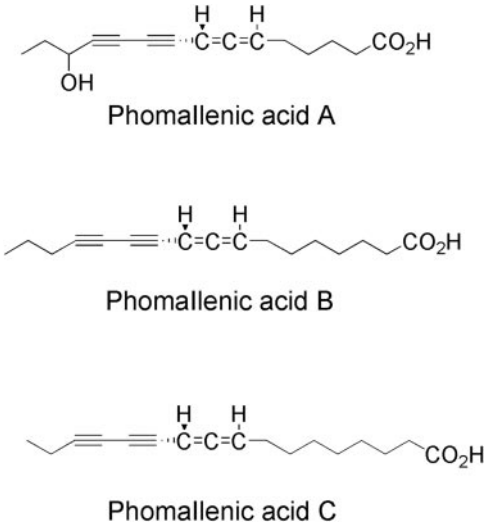

FIG. 4. Structures of phomallenic acids A, B, and C.

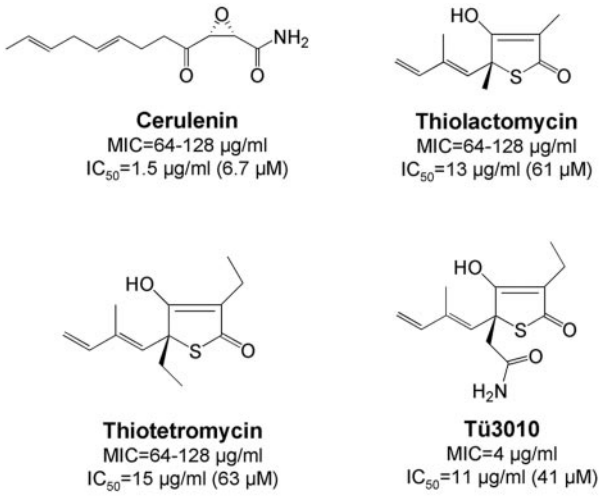

FIG. 5. Structures and activities of known FabH and FabF inhibitors. IC_{50} values were obtained from the FASII assay using wild-type *S. aureus* (MB2985) FASII enzymes. The MICs are also representing wild-type *S. aureus* (MB2985) results.

acids and contains a ω3 hydroxy group. The other two phomallenic acids, B and C, possess a two- and three-carbon-longer chain than A, respectively. They differ by a methylene group, phomallenic acid B being the smaller of the two. Although these three analogs share very similar chemical structures, their activities differ by up to 64-fold. Phomallenic acids A, B, and C displayed MICs of 250, 12.5, and 3.9 μg/ml, respectively, against *S. aureus*. As expected, these compounds did not exhibit any MIC differences between wild-type *S. aureus* and MRSA, indicating no cross-resistance with methicillin. Phomallenic acids A, B, and C showed no activity against the wild-type *E. coli* parent strain (MIC > 250 μg/ml) or the *E. coli tolC* strain (data not shown). However, they had MICs of 31.3, 50, and 12.5 μg/ml, respectively, against the outer membrane-permeable *E. coli lpxC* strain. Phomallenic acid C showed the best overall antibacterial and FASII (IC_{50}s of 0.77, 13.4, 2.5, and 2.7 μg/ml for *S. aureus*, *E coli*, *H. influenzae*, and *B. subtilis*, respectively) activities among all three compounds. Against *H. influenzae*, phomallenic acid C had an MIC of 3.9 μg/ml while phomallenic acids A and B gave MICs of 62.5 μg/ml and 7.8 μg/ml, respectively. This trend was also evident against *B. subtilis* as well with phomallenic acid C exhibiting an MIC of 15.6 μg/ml. Phomallenic acid B showed an intermediate MIC of 31.3 μg/ml, and phomallenic acid A afforded the least inhibitory activity (MIC of 250 μg/ml). All data showed a good correlation between the biochemical FASII IC_{50} values and antibacterial activities that supports the possibility that the inhibition of fatty acid synthesis enzymes is responsible for the inhibition of bacterial growth.

Target confirmation using FASII gel elongation. Titration of all three compounds in a FASII gel elongation assay (Fig. 6B, C, and D) showed IC_{50} values consistent with the values obtained in the FASII (Flashplates) assay (Table 1). Without inhibitor, malonyl-ACP is almost completely converted to $C_{20:0}$-ACP and $C_{22:0}$-ACP. At a higher concentration of inhibitor, an accumulation of malonyl-ACP was observed, demonstrating that none of the three phomallenic acids blocks FabD, a malonyl-CoA:ACP transacylase. The intermediate concentrations of phomallenic acids produced partial inhibition and accumulations of $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$-ACP, indicating that the targets of inhibition are condensing enzymes in the FASII elongation cycle. Cerulenin, a FabF inhibitor, produced similar results (Fig. 6A) while triclosan, a FabI inhibitor, differed and *trans*-2-enoyl-ACP accumulates (24).

Whole-cell target selectivity of phomallenic acids. Purified phomallenic acids A, B, and C were titrated in the two-plate assay (Fig. 7). The minimum detection concentrations (MDCs) of phomallenic acid A on control and on *fabF* AS-RNA plates were 5 and 0.63 μg/ml, respectively. The MDCs of phomallenic acid B were 2.5 and 0.31 μg/ml, respectively, and MDCs of phomallenic acid C were 0.63 and 0.15 μg/ml, respectively. These results support the idea that phomallenic acids reach intercellular targets and selectively inhibit condensing enzymes to inhibit cell growth. In this assay, it has been noted that zone quality may be correlated with the MIC-to-MDC ratio. In general, if the zone quality is clear, the MDC on the control plate is about four- to eightfold lower than liquid MIC (e.g., phomallenic acids B and C). If zone quality is hazy, the MDC is 25- to 50-fold lower than the MIC (e.g., phomallenic acid A). Whole-cell labeling experiments (data not shown), however, do not show preferential inhibition of [^{3}H]glycerol incorporation into phospholipids over that of incorporation of other radiotracers (24). The reasons for this discrepancy are unclear and under investigation.

DISCUSSION

To discover antibiotics for a novel target, several approaches have been used in past decades. One approach is empirical screening, in which a whole-cell assay is used to find antibacterial activities first, followed by determination of the targets or modes of action to guide further chemical modifications. Another approach is in vitro target-based screening, in which a cell-free system is used to find inhibitors for an essential enzyme (or enzymes in a pathway) followed by determination of the spectrum of antibacterial activity. However, there have been few successes with the latter approach because of the

TABLE 1. Biological activities of phomallenic acids[a]

Organism	Strain	Assay	Activity of phomallenic acid (μg/ml)		
			A	B	C
S. aureus	MB2985 (WT)	MIC	250	12.5	3.9
	MB5393 (MRSA)	MIC	250	12.5	3.9
	MB2985 (WT)	FASII (IC_{50})	22	3.4	0.77
E. coli	MB4827 (WT)	MIC	>250	>100	>100
	MB4902 (*lpxC*)	MIC	31.3	25	12.5
	MB4827 (WT)	FASII (IC_{50})	84	20	13.4
H. influenzae	MB4572	MIC	62.5	7.8	3.9
	MB4572	FASII (IC_{50})	159	11.9	2.5
B. subtilis	MB410	MIC	250	31.3	15.6
	MB410	FASII (IC_{50})	50	5.3	2.7

[a] FASII assay was described in Materials and Methods, and IC_{50} values for *S. aureus* MB2985 (WT) and *E. coli* MB4827 (WT) were similar to those for MB5393 (MRSA) and MB4902 (*lpxC*), respectively (data not shown). Experiments were done twice in duplicate. WT, wild type.

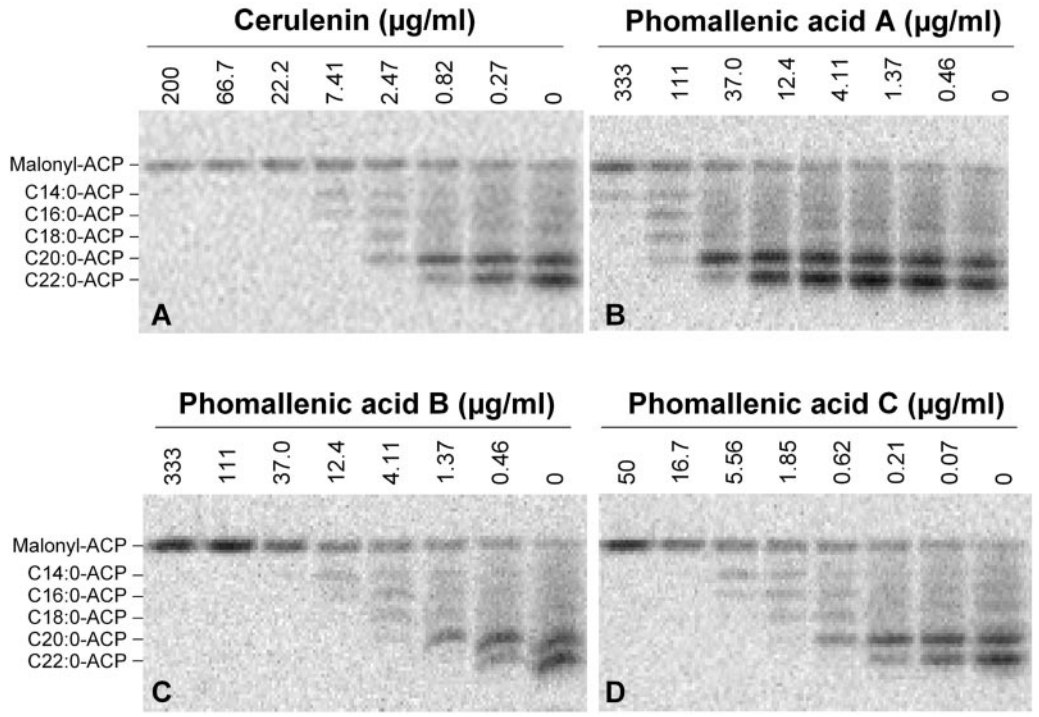

FIG. 6. FASII gel elongation assay on phomallenic acids. The FASII reaction was performed with serial dilutions of cerulenin (A), phomallenic acid A (B), phomallenic acid B (C), and phomallenic acid C (D) using *S. aureus* FASII enzymes as described in Materials and Methods. The reaction used 4 μM of [^{14}C]malonyl-CoA (60 mCi/mmol) and 20 μM of lauroyl-CoA ($C_{12:0}$) as substrates. Of each sample, 10 μl was directly applied to and resolved by a 16% polyacrylamide gel containing 4 M urea. The gel was blotted to a polyvinylidene difluoride membrane and visualized by using a PhosphorImager. Similar experiments were repeated twice with reproducible results.

variation in the ability of the compounds to reach their intracellular targets due to poor penetration (50) and/or active efflux (27, 33). The benefit of the whole-cell screening approach is that it ensures antibacterial activity; however, empirical screening may yield toxic compounds. Thus, over the years many target-based whole-cell screening methods have been developed to preferentially find certain types of inhibitors. Such screens may compare the responses of paired strains, such as a drug-resistant strain and its parent strain, or a wild-type strain and a strain overexpressing a selected target. Other whole-cell screens have monitored changes in cell morphology or specific phenotypes caused by blockage of a particular pathway or measured expression of a reporter gene which is directly or indirectly regulated by essential intracellular proteins.

The application of antisense technology to identify essential genes in *S. aureus* (18) and the expression of *fabF* antisense RNA to specifically increase sensitivity to cerulenin (8) were described previously. However, the mechanism by which antisense mRNA lowers gene expression was not clear. General speculations are that (i) antisense may target the genomic DNA, which terminates transcription by interfering with polymerase operation, or (ii) it may target mRNA to block translation machinery or (iii) when antisense RNA binds to mRNA, enzymes (such as RNase III) endonucleolytically cleave double-stranded RNA (32) and cleaved mRNA might be unstable and degraded nonspecifically by other RNases. Our data supported the last hypothesis but had somewhat unexpected results. The *fabF* antisense RNA, when overexpressed in *S. aureus*, targets the *fabF* mRNA, causing a complete degradation of the 5′ portion while keeping the 3′ portion intact, indicating that transcription is not affected while translation is affected solely because of degradation of the mRNA transcript. Furthermore, genes upstream (5′) of the antisense targeting site in the same operon are equally affected while downstream

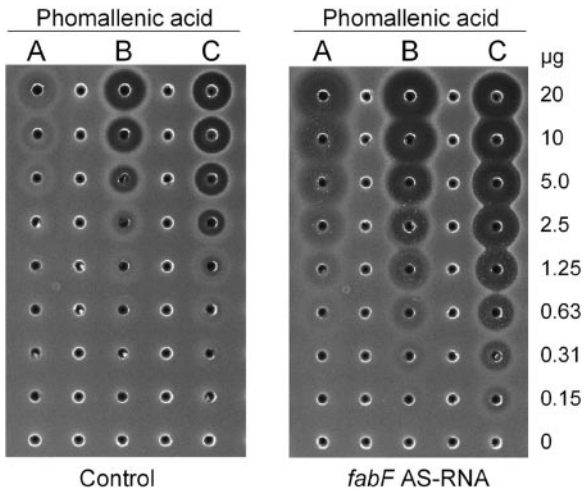

FIG. 7. Two-plate assay of purified phomallenic acids. Two-plate assays were performed with serial dilutions of phomallenic acid A (lane A), phomallenic acid B (lane B), and phomallenic acid C (lane C). Two-plate preparations and assays were detailed in Materials and Methods.

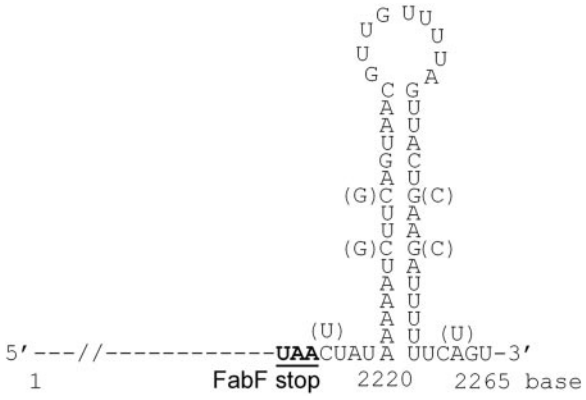

FIG. 8. *S. aureus*. RN4220 and RN450 sequences of the stem-loop structure at the 3′ end of the *fabH* and *fabF* mRNA were determined in this study. The differences in nucleotides from *S. aureus* COL, Mu50, N315, MW2, MRSA252, and MSSA476 are shown in parentheses. A 16-base inverted repeat (stem) with a loop (bases 2220 to 2260) found four nucleotides after the *fabF* stop codon.

(3′) genes might not be affected if an intact ribosome binding site is present. Why is the 5′ portion of the transcript degraded but not the 3′ portion? The presence of a 3′ terminator has been postulated to stabilize mRNA against 3′-to-5′ degradation (9). Indeed, the 3′ untranslated region of FabH/F mRNA contains a terminator-candidate stem-loop structure immediately after the *fabF* stop codon (Fig. 8). Our hypothesis is that, in *S. aureus* after antisense RNA binds mRNA, double-strand-specific RNase III (15) selectively cleaves the antisense RNA-mRNA hybrids (44) and the 5′ portion of the mRNA is degraded possibly by a *B. subtilis* (not *E. coli*) PNPase-like enzyme(s) (4) while the 3′ portion remains intact due to protection by the terminator stem-loop.

Recently, conditional expression of antisense RNA in cells has been found to alter cell sensitivity to selective inhibitors (8, 17), leading to the possibility of new, more sensitive target-based whole-cell assays. This new approach to directly screen natural product broths has been developed using a combination of antisense and agar-diffusion technologies, as well as a biochemical pathway assay. As a proof of concept, all known natural product inhibitors for the FASII condensing enzymes including thiolactomycin, thiotetromycin, Tü3010 from actinomycetes, and cerulenin from fungal fermentations were identified, using this mechanism-based screen. In addition, new fungal products, phomallenic acids, were also identified. These three phomallenic acids showed target selectivity in the gel elongation assay and in the target-based two-plate assay. Although this selectivity together with good correlation between IC_{50} values of FASII assay and antibacterial activity supports the idea that phomallenic acids inhibit cell growth through inhibition of condensing enzymes, this has not yet been confirmed by whole-cell labeling experiments. It is possible that the specific labeling conditions used (20-minute incorporation of [^{3}H]glycerol) obscure specificity, and this needs further studies.

In a gel elongation assay (Fig. 6), lauroyl-CoA ($C_{12:0}$) was used as a substrate. If phomallenic acids inhibited elongation enzymes rather than condensation reactions, they would not stop the addition of the first two carbons to form the 14 carbons of β-ketoacyl-ACP, β-hydroxyacyl-ACP, or *trans*-2-enoyl-ACP. However, no accumulation of any of these intermediate products was seen, which is similar to cerulenin but different from triclosan (24), further demonstrating that phomallenic acids target the FabH and FabF enzymes. From Fig. 6 and Fig. 7C in reference 24 as well as Fig. 4 in reference 49, data showed that, without any inhibitors, the majority of intermediate products accumulated were not the FabG substrate (β-ketoacyl-ACP), FabZ substrate (β-hydroxyacyl-ACP), or FabI substrate (*trans*-2-enoyl-ACP) but rather the FabF/B substrate ($C_{2n:0}$-ACP, $n \geq 2$), suggesting that the condensation step in the elongation cycle is the rate-limiting step in both *E. coli* and *S. aureus*. A recent publication (35) showed that *S. aureus* FabH has a significantly larger primer binding pocket and different substrate selectivity than does *E. coli* FabH, which is consistent with our observation (data not shown) that *S. aureus* FabH is able to use long-chain acyl-CoAs as substrates. The *S. aureus* FASII assay in this study involves both condensing enzymes, FabH and FabF. Therefore, phomallenic acids are dual inhibitors that target both FabH and FabF in *S. aureus*.

Although the three phomallenic acid analogs have similar chemical structures, the compound with longer hydrophobic chains showed higher activity in both the biochemical and the antibacterial assay. Phomallenic acid C showed good antibacterial activity, about 20-fold better than that of thiolactomycin and cerulenin against *S. aureus*, and has a good spectrum against key pathogens including MRSA, *H. influenzae*, and *B. subtilis*.

Since its discovery in 1982 as an antibacterial agent with low toxicity due to bacterial target selectivity, thiolactomycin has been proposed as a promising antibacterial lead and significant chemical modifications have been made (7, 10, 19, 20, 25, 29, 38, 40, 41). Phomallenic acids, along with other FabH and FabF/B inhibitors (5, 14, 22, 24), provide additional opportunities for development of new antibiotics with novel modes of action to overcome the threat posed by emerging resistant pathogens.

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